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GPR54 RECEPTOR AGONIST AND ANTAGONIST USEFUL FOR THE TREATMENT OF GONADOTROPIN RELATED DISEASES

FIELD OF THE INVENTION

The invention relates to GPR54 receptor agonist and antagonist that are useful for the treatment of gonadotropin related diseases, as well as in the diagnostic field. These compounds will find application in many pathologies, known to be dependant upon GnRH or LH/FSH secretion.

BACKGROUND OF THE INVENTION

The integrity of the gonadotropic axis leads to normal sexual differentiation during fœtal life, normal puberty and therefore to normal fertility. Genetic defects leading to isolated impuberism and infertility have been described in genes encoding for known proteins as GnRH receptor, gonadotropins, gonadotropin receptors and steroidogenic enzymes.

Hypogonadisms related to deficiency of GnRH or LH/FSH synthesis are called hypogonadotropic hypogonadism. Cases of congenital isolated hypogonadotropic hypogonadism are classified into 2 categories: those associated with anosmia (Kallmann syndrome) and those apparently isolated. Mutations and deletions of the KAL-1 gene have been observed in many cases of the X-linked form of Kallmann syndrome. Cases of hypogonadotropic hypogonadism without anosmia were considered as idiopathic until the recent description of mutations of the GnRH receptor gene.

Inhibition or activation of the gonadotropic axis are useful schemes for the treatment of hormones-related diseases, such as gonadotropin deficiency, precocious puberty, and some types of cancer (e.g. prostate and breast cancers) and useful to manage in-vitro fecundation. For the time being, only the GnRH receptor is known to play a role in regulating LH and FSH secretion, although it is possible that GnRH receptor may

itself has regulation effects in cancer independent of LH and FSH.

GPR54 was initially described as an orphan receptor homolog to galanin receptor. Recently, a ligand acting on GPR54 bas been described. Katani et al as well as Ohtaki et al analysed placental extracts for peptides activating GPR54. Muir et al used a library of 1500 putative ligands. The best agonists displayed a similarity to a 54 amino acids peptide derived from the KiSS-*1* protein (also found were peptides of 14, 13 and even fewer amino acids). This peptide corresponds to the predicted proteolytic processing of KiSS-*1* at dibasic and dibasic/amidation sites, and have been named Kisspeptins. GPR54 stimulation by this 54 amino acid peptide results in the activation of phospholipase C by coupling to a Gq protein. It was also determined that GPR54 was mainly present in pituitary and placenta, and that Kisspeptins are high affinity agonists of the GPR54 receptor. Kotani et al. concludes that tissue distribution suggested that GPR54 might be implicated in various hormonal functions, a hypothesis supported by the demonstration that KISS-1 derived peptides stimulate oxytocin release in rats.

Human and rat GPR54 genes have been fully disclosed in many publications, referred to in the above Kotani, Ohtaki and Muir publications. *GPR54* gene is formed of 5 exons. GPR54 has also sometimes been named AXOR12 or SNORF11.

WO-A-2003003983 discloses a method of treating an abnormality which comprises administering to the subject an amount of a SNORF11 (GPR54) receptor agonist. Examples of such agonists that are given include KISS-1 peptide fragments. It is also indicated that the SNORF11 (GPR54) receptor may serve as a tool for designing drugs for treating various pathological conditions, including, cancers, sexual/reproductive disorders, benign prostatic hypertrophy. The sole example given relates to pain.

US-A-20020106766 discloses the rat AXOR12 gene sequence and potential uses of agonists and antagonists

of the receptor. There is however no working example of any pathology in this document.

EP-A-1126028 (naming Ohtaki as an inventor) discloses GPR54 gene, encoded protein, ligands and potential uses, which include diagnostic and screening uses. Rat and human GPR54 proteins and coding sequences are disclosed in SEQ ID NO:1 and 5, respectively.

However, none of the above documents teaches or suggests the present invention.

SUMMARY OF THE INVENTION

The invention shows that GPR54 is a new hormonal system playing an important and previously unsuspected role in the physiology of the gonadotropic axis.

Hence, the invention offers a further route for defining new pharmacological strategies to activate or inhibit the gonadotropic axis and investigating gonadotropic hormones related pathologies.

The invention thus provides an agonist or antagonist of the GPR54 receptor for its use for treating a gonadotropin related disorder.

In one embodiment, the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, or a partial (functional) protein thereof, or an ester, amide or salt thereof.

In another embodiment, the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

In another embodiment, the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

The agonist or antagonist of the invention is useful for its use for treating hypogonadotropic hypogonadism, LH and/or FSH related disorders, gonadotropin-estradiol/testosterone-dependent related cancers and/or gonadotropin related reproductive disorders.

The invention also provides a ligand of the GPR54 receptor for its use for diagnosing a subject's gonadotropin abnormality, such as hypogonadotropic hypogonadism.

In one embodiment, the ligand of the invention binds to the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

In another embodiment, the ligand of the invention binds to the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

The invention also provides a method for screening a compound that affect the gonadotropic axis comprising the

step of assaying the compound in the presence of a GPR54 receptor.

In one embodiment, the screening method of the invention aims at screening for a compound that affects (for example effects) the LH and/or FSH secretion.

In one embodiment, in the screening method of the invention, the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, or a partial protein thereof, or an ester, amide or salt thereof.

In another embodiment, in the screening method of the invention, the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

In another embodiment, in the screening method of the invention, the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

The invention provides novel proteins, i.e. the proteins shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398 as well as the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P, together with antibodies specific to these.

The invention also provides an agonist or antagonist of the GPR54 receptor for its use as an addition to a treatment for the stimulation of ovulation by GnRH.

In another embodiment the agonist or antagonist of GPR54 positively modulates the GnRH effect on LH synthesis stimulation. In another embodiment the GPR54 protein is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, or a partial protein thereof, or an ester, amide or salt thereof. Additionally GPR54 can be the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398 or the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P

In another embodiment the invention also provides a composition comprising GnRH and agonists or antagonists to GPR54. In another embodiment the ratio of the GnRH to the agonist or antagonist of GPR54 is in the range 10:1 to 1000:1 in Molar concentration. In another embodiment the agonist is the fragment 45-54 of Kiss-1.

BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCES

Figure 1 shows the pedigrees of the two affected families.

Figure 2 is a GnRH (100 µg/iv) test performed in the propositus of family 2

Figure 3 is the amino acid sequence of human *GPR54*. Boxes highlight putative transmembrane domains. The site of the deletion observed in affected individuals is indicated by an arrow. The deleted protein sequence is in italics.

Figure 4: Functional characterisation of the L102P mutated receptor. Wild type and mutated plasmids were transfected within HEK293T cells. Inositol Phosphate accumulation was measured 48 hours after the transfection. Transfection with an empty expression vector (pcDNA) was used as a control.

Figure 5: Kiss-1 effects on LH secretion: Two male rat pituitaries were pooled within each perifused chambers (four chambers for each condition). A buffer was perifused within each chamber with a flow of 0.1 ml/mn. Samples were collected every 5 minutes. Stimulations were performed three times at 50 minutes interval. This figure shows the result from four different chambers (two chambers with GnRH alone being chambers 5 and 6 and two chambers with GnRH and Kiss-1 (fragment 45-54, being a functional truncation of Kiss-1) being chambers 9 and 10), Open boxes indicate the time of the agonists injection. No stimulation was observed for kiss-1 alone.

SEQ ID NO:1 is the sequence of human GPR54 (gene and encoded protein) while SEQ ID NO:2 is the sequence of the protein. SEQ ID NO:3 is another sequence for the protein of human GPR54 showing a polymorphic variation at position 364 according to which leucine is replaced by histidine (corresponding to the GPR54 shown in figure 3).

DETAILED DESCRIPTION OF THE INVENTION

As indicated above, the inventors have found that GPR54 plays an important and previously unsuspected role in the physiology of the gonadotropic axis. The present invention describes a new genetic etiology for impuberism. It shows that alteration of GPR54 (KiSS-1 peptide receptor) plays an important and previously unsuspected role in the initiation of puberty. Therefore, loss of function of GPR54 leads to hypogonadotropic hypogonadism.

This was demonstrated by sequencing the GPR54 nucleotide sequence of affected patients, where the patients were suffering from hypogonadotropic hypogonadism (impuberism). GPR54 was chosen as candidate gene as it is localized in the region of interest defined by genome mapping in a very informative family. A homozygous deletion within intron 4 and exon 5 of the GPR54 gene was found in all affected siblings in one family. In a second family showing a recessive transmission, a loss of function homozygous point mutation of GPR54 was found within exon 1.

These findings will have useful applications in diagnostic and drug design, in pathologies that are related to GnRH or gonadotropin secretion.

The GPR54 receptor proteins and the like are useful, among other things: (1) for determination of an agonist or antagonist to the GPR54 receptor, where these agonist and antagonist compounds would be useful in gonadotropin-related diseases, (2) for screening of compounds (agonist, antagonist, etc.) that alter the binding property between GPR54 and a ligand, whereby the screened compound would then be useful for the treatment of gonadotropin-related diseases and (3) for determination of an agonist or antagonist to the GPR54 receptor where these agonist and antagonist compounds would be useful in assisted reproduction (4) for diagnosing gonadotropin-related diseases, as a genetic diagnostic agent or (5) for determination of a compound leading to perform

dynamic hormonal tests of the gonadotropic axis during diagnosis procedure.

Gonadotropin-related diseases include those pathologies involving malfunction in the LH and/or FSH secretion, hypogonadotropic hypogonadism, precocious puberty, uterine leiomyomas), severe endometriosis, hyperandrogenism, menometrorrhagia, catamenial disorders and endometrial hyperplasia, and prostate and breast cancers known to be LH-dependent estradiol/testosterone-dependent disorders.

The administration of agonists or antagonists of GPR54 leading to the activation or inhibition of the gonadotropic axis, can be suitable for treating the gonadotropin-related diseases. Agonists or antagonists of GPR54 can be indeed useful for stimulating or inhibiting GnRH or LH/FSH synthesis.

Agonists of GPR54 are herein defined as compounds able to activate GPR54.

Technologies related to gene therapy are known to the skilled man. Said agonists can also consist in compounds that mimic the natural ligand of GPR54, such as kiss-1 peptide, kisspeptins, or derived peptides thereof. Suitable agonists to the receptor GPR54 can be specific or not, and can consist in natural or synthetic compounds. These agonists include but are not limited to Kiss-1 peptide itself, Kiss-1 peptide fragments, kisspeptins, or salt thereof, but can also include those described in WO-A-200300398, US-A-20020106766, and EP-A-1126028. Thus the invention proposes the use of an agonist of GPR54 for treating diseases related to a deficiency of GnRH or LH/FSH.

Antagonists of GPR54 are herein defined as a compounds able to inhibit the activation or the expression of GPR54.

Compounds capable of inhibiting the activation of GPR54 include in particular those able to interact with natural agonists of GPR54, such as kiss-1 peptide, to inhibit the binding of said agonists, or to inhibit the

activation of GPR54 resulting from said binding. For instance, a inhibitor of activation of GPR54 can consist in an antibody directed to Kiss-1 peptide, or kisspeptins, which impedes the binding of said Kiss-1 peptide, or said kisspeptins to GPR54.

Inhibitors of the expression of GPR54 include for instance antisense oligonucleotides, or interfering RNAsi, or ribozymes, targeting the GPR54 gene .

Antisense nucleic acids that can inhibit replication or expression of the GPR54 receptor protein gene can inhibit RNA synthesis or the function of RNA, or can regulate/control the expression of the receptor protein gene via the interaction with RNAs associated with the receptor protein. Antisense nucleic acids are useful for regulating and controlling the expression of the receptor protein gene in vivo and in vitro, and are also useful for the treatment and diagnosis of the diseases described above.

Technologies related to such antisense RNAs and gene therapies are known to the skilled man.

Said agonists or antagonists can be administered by themselves, or mixed with suitable carriers or excipients.

Said agonists and antagonists can be further identified by the screening methods described hereinafter.

The screening methods of the invention can be carried out according to known methods. Those depicted in EP-A-1126028, WO-A-2003003983 and US-A-20020106766 are suitable.

The screening method may measure the binding of a candidate compound to the receptor, or to cells or membranes bearing the receptor, or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound. Alternatively, a screening method may involve measuring or, qualitatively or quantitatively, detecting the competition of binding of a candidate compound to the receptor with a labelled

competitor (e.g., agonist or antagonist). Further, screening methods may test whether the candidate compound results in a signal generated by an agonist or antagonist of the receptor, using detection systems appropriate to cells bearing the receptor. Antagonists can be assayed in the presence of a known agonist and an effect on activation by the agonist by the presence of the candidate compound is observed. Further, screening methods may comprise the steps of mixing a candidate compound with a solution comprising a GPR54 receptor, to form a mixture, and measuring the activity in the mixture, and comparing to a control mixture which contains no candidate compound. Competitive binding using known peptide agonist such as the KISS peptides mentioned above is also suitable.

Assays techniques are known in the art and the skilled man may revert to publications to that effect, such as the mentioned patents, e.g. EP-A-1126028, WO-A-2003003983 and US-A-20020106766.

The GPR54 receptor of the present invention may be employed in conventional low capacity screening methods and also in high-throughput screening (HTS) formats.

Screening kits can then be manufactured using known techniques.

Once screened and identified, the useful compounds are conventionally used as pharmaceutical compositions.

The diagnostic methods may be carried out using the methods disclosed in EP-A-1126028. Notably, antibodies can be used, where the antibodies, monoclonal or polyclonal can be manufactured by publicly known methods.

Laboratory methods for preparing monoclonal antibodies are well known in the art. Monoclonal antibodies (mAbs) may be prepared by immunizing purified mutated GPR54 protein into a mammal, e.g. a mouse, rat, human and the like mammals. The antibody-producing cells in the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal

antibodies are utilized as a source of the desired monoclonal antibody. Phage display technology is also a useful technology for the production of mAbs.

Antibodies according to the invention are designed to be specific to the mutated form of GPR54 protein, ie; that are capable of distinguishing between a mutated form of GPR54 protein and the wild-type GPR54 protein.

Especially, antibodies shall permit to identify the GPR54 protein lacking its transmembrane domains 6 and 7, or having the point mutation L102P.

Other ligands can be used, as long as they allow recognition the presence (or absence) of (part) of the GPR54 protein.

Diagnostic kits involving nucleic acids or protein assays can then be manufactured using known techniques.

The GPR54 protein useful in the present invention is one that has an amino sequence identical or substantially similar to the one depicted in SEQ ID NO:2 or SEQ ID NO:3. Preferably, the sequence includes an amino acid sequence having at least about 70% homology, preferably at least about 80% homology, more preferably at least about 90% homology, most preferably at least about 95% homology, to the protein sequence represented by either SEQ ID NO:2 or SEQ ID NO:3. Partial peptides can be used.

The instant invention is not limited to human GPR54, but can be applied to any other mammals, including those useful in the agricultural field, it being understood that the GPR54 is the one corresponding to the mammal of interest.

Specific examples include the protein corresponding to the polypeptide from residue 247 to 398 of SEQ ID NO:2 (hereinafter deleted or truncated GPR54 protein) or the polypeptide shown in SEQ ID NO:2 with the mutation L102P (proline substituted for leucine) (hereinafter 102-mutated GPR54 protein) or the polypeptide shown in SEQ ID NO:3 (bearing a polymorphism such that leucine is replaced by histidine at position 364 compared to SEQ ID NO:2) or the polypeptide shown in SEQ ID NO:3 with the

mutation L102P or the polypeptide from residue 247 to 398 of SEQ ID NO:3.

The receptor protein of the present invention which can be used may be a protein comprising (i) an amino acid sequence represented by SEQ ID NO:2 or SEQ ID NO:3, or the truncated or 102-deleted corresponding protein, in which one, two, or more amino acids (preferably 1 to 30 amino acids, more preferably 1 to 10 amino acids, most preferably 1 or 2 amino acids) are deleted; (ii) an amino acid sequence represented by SEQ ID NO:2 or SEQ ID NO:3, or the truncated or 102-deleted corresponding protein, to which one, two, or more amino acids (preferably 1 to 30 amino acids, more preferably 1 to 10 amino acids, most preferably 1 or 2 amino acids) are added; (iii) an amino acid sequence represented by SEQ ID NO:2 or SEQ ID NO:3, or the truncated or 102-deleted corresponding protein, in which one, two, or more amino acids (preferably 1 to 30 amino acids, more preferably 1 to 10 amino acids, and most preferably 1 or 2 amino acids) are substituted by other amino acids; and (iv) a combination of the above amino acid sequences.

The partial peptide of the GPR54 receptor protein of the present invention (hereinafter sometimes referred to as the partial peptide) may be any partial peptide, so long as it constitutes a part of the peptide portions of the receptor protein described above retaining binding properties. Examples of such partial peptides include site, which is exposed outside cell membranes among the receptor protein and retain the receptor binding activity or the transmembrane domains. These domains are identified in figure 3.

An example is a peptide containing a region which is analyzed to be an extracellular area (hydrophilic region or site) in a hydrophobic plotting analysis.

It is also possible to have partial peptides fused together.

The number of amino acids in the partial peptide of the present invention is at least 20 or more, preferably

50 or more, more preferably 100 or more, in terms of the constructive amino acid sequence of the GPR54 receptor protein described above.

Esters, amides or salts can also be used, as disclosed in EP-A-1126028.

The receptor protein of the present invention may be manufactured in accordance with a publicly known method for purification of a receptor protein from human or other mammalian cells or tissues. Alternatively, the receptor protein of the present invention or salts thereof may also be manufactured by culturing a transformant containing DNA encoding the receptor protein of the present invention, as will be later described. Furthermore the receptor protein of the present invention or salts thereof may also be manufactured by known methods for synthesizing proteins.

Finally, the invention provides two specific proteins, one being truncated or deleted, and the other being mutated. The invention also provides the polynucleotides (purified) encoding said proteins, a vector comprising said polynucleotide and a host cell comprising the vector.

Also within the ambit of the invention is the antisense nucleic acid, as well as the gene therapy using the above GPR54 receptor.

The G protein coupled receptor may be used not only for administration of antagonists or agonists of the receptor, but also for gene therapy by transfer of the receptor gene into the body (or certain specific organs such as the hypophysis) or by transfer of the antisense nucleic acid to the receptor gene.

Antisense nucleic acids that can inhibit replication or expression of the GPR54 receptor protein gene can inhibit RNA synthesis or the function of RNA, or can regulate/control the expression of the receptor protein gene via the interaction with RNAs associated with the receptor protein. Antisense nucleic acids are useful for regulating and controlling the expression of the receptor

protein gene in vivo and in vitro, and are also useful for the treatment and diagnosis of the diseases disclosed above.

Technologies related to such antisense RNAs and gene therapies are known to the skilled man.

EXAMPLES.

A consanguineous family (family 1) with 5 affected sibs was investigated (see Fig. 1). The propositus was a 20-year old male referred for impuberism. He had typical signs of hypogonadism with small testes (28x17 mm), sparse pubic hair (P3) and a penis of 7 cm. His bone age was retarded at 15.0 years. He had a normal sense of smell and showed no abnormal eye movements, no colour blindness and no renal or cranio-facial abnormalities. His weight and height were 54 kgs and 152 cm respectively. Three brothers presented similar clinical signs. A sister had a partial hypogonadism. At 16, she had partial breast development and she reported a single episode of uterine bleeding. Hormone assays (Table 1) showed low plasma testosterone in boys and low plasma oestradiol in the sister accompanied by low plasma gonadotropin levels. All sibs had a partial or a blunted response to GnRH (100µg IV). One other brother and two other sisters had a normal pubertal development. The parents were first cousins and have had normal pubertal development. Table 1 below gives the hormonal status of the affected patients of family 1

Patient	Age	Bone age	Plasma Testosterone (ng/dl)	Plasma Oestradiol (pg/ml)	Plasma LH (mU/ml)	Plasma FSH (mU/ml)	GnRH test	
							LH	FSH
III.2	21	15	26	-	1.5	0.5	3.6	1.7
III.3	20	15	19	-	1.5	0.5	1.4	1.5
III.4	19	-	5	-	1.1	4.1	1.9	4.1
III.6	18	-	-	17	2.0	3.4	11.8	6.4
III.7	14	11	5	-	2.6	1.8	3.4	2.6

The chronological age and the bone age are indicated. Normal values: (males) LH, 1.0-5.0 IU/ml, FSH 0.9-5.7 IU/ml), Testosterone 260-690 ng/dl; (Females) LH, 1.1-5.4 IU/ml, FSH 2.3-6.0 IU/ml, Oestradiol (early follicular phase) 25-90 pg/ml. The GnRH test was performed by intra venous administration of 100 µg of GnRH. The highest values observed for plasma LH and FSH are reported.

The second family (family 2) was a consanguineous family originated from Kurdistan. The propositus was a 27 years old woman referred for primary amenorrhea. She had a normal breast and pubic hair development. She had a normal sense of smell. Ultrasonography showed a small uterus with thin infantile endometrium. Ovaries were small with several small immature follicles. Plasma oestradiol is low accompanied by normal plasma gonadotropins. All other anterior pituitary hormone plasma levels were in normal range. The GnRH test performed with 100 µg/IV showed a normal response for the FSH and an explosive response for LH (see figure 2). LH pulsatility showed low amplitude peaks but normal frequency. A pulsatile GnRH pump administration led to a normal pregnancy.

For both family sibs, genomic DNA was isolated from peripheral lymphocytes following standard methods.

The 5 exons of the *GPR54* gene were amplified by PCR with 20 to 100 ng of genomic DNA. The following primers were used:

Exon 1: Forward : GGGCGGCCGGGAGGAGGA
Reverse : CCGGGACGGCAGCAGGTG
Exon 2: Forward : GCCCAGCGCCCGCGCATC
Reverse : GTCCCCAAGTGCGCCCTCTC
Exon 3: Forward : CAGGCTCCCAACCGCGCAG
Reverse : CGTGTCCGCCTTCTCCCGTG
Exon 4: Forward : CTTCATCCTGGCTTGTGGCAC
Reverse : CTTGCTGTCCTCCCACCCAC
Exon 5: Forward : GCCTTTCGTCTAACCACCTTC
Reverse : GGAGCCGCTCGGATTCCCAC

Amplification was performed for 30 cycles with Yellow Taq (Eurogentec) in 1.5 mM $MgCl_2$, with 0.1 µM of each primer and 5% DMSO. The annealing temperatures were of 60° for exons 1, 3, 4, 5 and of 66° for exon 2. The PCR products were directly sequenced with BigDye dideoxyterminator cycle sequencing kits and the 3100 sequencer (Applied Biosystems) using the same primers. To genotype all members of the family, the PCR products of exon 5 were analyzed by electrophoresis in 2% agarose gel.

Upon study of the *GPR54* gene, it was observed in affected individuals a homozygous deletion of 155 base pairs lying between intron 4 (nucleotide -13 when numbering from the 3' end of the intron 4) and exon 5 (nucleotide 142 of the exon 5, corresponding to nucleotide 880 of the cDNA). The deletion reported in family 1 removes the splicing acceptor site of intron 4-exon 5 junction. It thus leads to the absence of the normal protein sequence downstream from residue 247 (Fig. 3). The deleted receptor is truncated within the third intracellular loop thus lacking transmembrane domains 6 and 7 (Fig. 3). All affected patients were homozygous for this deletion. Both parents as well as unaffected sib III.5 were heterozygous. Unaffected sib III.1 was homozygous for the wild type sequence. The deletion was absent in 50 control subjects.

In family 2 showing a recessive transmission, a homozygous point mutation was found within exon 1. This mutation substituted a proline for a leucine (L102P) at the N-terminal extremity of the first extracellular loop.

The L102P mutation was reproduced by PCR in-vitro mutagenesis and sub-cloned within a human GPR54 expressing vector (pGPR54_L102P). The functional characterisation of the L102P mutated receptor was performed after transient transfection of the wild type and mutated L102P plasmid within HEK293T cells. The activation of G-protein activated phospholipase C-β, was

evaluated after stimulation by different concentrations of Kiss1 peptide (decapeptide 45-54, or fragment 45-54 being a functional truncation of Kiss-1). Inositol phosphate accumulation was measured after [H3] inositol cell labelling and ion exchange chromatography purification. Dose response clearly showed that substitution of leucine 102 by a proline inactivates phospholipase C stimulation in HEK293T cells (see figure 4). L102P mutation is thus a loss of function mutation of GPR54. This demonstrates that gonadotropic deficiency observed in family 2 is also due to the inactivation of GPR54 function by L102P mutation.

GPR54 is thus involved in the regulation of the LH and FSH synthesis. There is strong evidence that the Kiss-1 peptide is the GPR54 ligand involved in this physiological process as evidenced by the characterisation of a genetic abnormality in a hypogonadotropic hypogonadism patient born from first cousin parents. A duplication in tandem of 20 nucleotides localized at the 3' end of exon 3 of the human kiss-1 gene has been found. This duplication does not change the C-terminal end of the peptide but is likely to disturb the mRNA stability or translation. This duplication was not found in 400 chromosomes which demonstrates that it is not a polymorphism.

Physiological experiments have confirmed that the Kiss-1 peptide is the GPR54 ligand. GPR54 is expressed within the pituitary. LH plasmatic pulsatility measured in the propositus of family 2 has suggested that GPR54 inactivation defect occurs at the pituitary level. An approach using rat perifused pituitaries was used to study the effects of Kiss-1 stimulation on pituitary LH secretion. Rat pituitaries were in-vitro perifused with different concentrations of Kiss-1, GnRH or with a combination of Kiss-1 with GnRH (see figure 5). LH levels were measured at 5 minute intervals. A single peak was

observed immediately after the injection of the GnRH and then the levels returned to the base line level. This stimulation was marked after the second GnRH injection as expected (see chamber 5 and 6). Such priming effect was already described for the GnRH. After stimulation with both GnRH and Kiss-1 (peptide 45-54 or fragment 45-54, a functional truncated variant of Kiss-1), one peak was observed immediately as described for the GnRH alone. However, the LH levels did not return to the basal line level (see chambers 9 and 10). The total LH output (area under the curve) was clearly increased in chambers stimulated by both agonists when compared with GnRH alone (chambers 5 and 6). No stimulation was observed with Kiss-1 alone, whatever the concentrations used (10^{-10}, 10^{-8}, 10^{-6}). This demonstrates that Kiss-1 extend the effect of the GnRH on the LH synthesis stimulation by pituitaries.

It has thus been demonstrated that Kiss-1 positively modulates the GnRH effect on LH synthesis stimulation. Kiss-1 may be classified as a strong modulator of the GnRH effect as the functional integrity of its receptor GPR54 is required for a normal activation of the gonadotropic axis. The perifused pituitaries approach shows that Kiss-1 directly acts on pituitary cells. These results provide evidence that agonists or antagonists of GPR54 can modulate LH synthesis stimulation by the GnRH.

In compositions comprising GnRH and suitable agonists or antagonists of GPR54, suitable ratios of GnRH to the agonist/antagonist range between 10:1 to 1000:1 in Molar concentration.

CLAIMS

1. An agonist or antagonist of the GPR54 receptor for its use for treating a gonadotropin related disorder.

2. The agonist or antagonist of claim 1, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, or a partial protein thereof, or an ester, amide or salt thereof.

3. The agonist or antagonist of claim 1 or 2, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

4. The agonist or antagonist of claim 1 or 2, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

5. The agonist or antagonist of any one of claims 1 to 4 for its use for treating a gonadotropin related reproductive disorder.

6. The agonist or antagonist of any one of claims 1 to 4 for its use for treating hypogonadotropic hypogonadism.

7. The agonist or antagonist of any one of claims 1 to 4 for its use for treating LH and/or FSH related disorders.

8. The agonist or antagonist of any one of claims 1 to 4 for its use for treating gonadotropin-estradiol/testosterone-dependent related cancers.

9. A ligand of the GPR54 receptor for its use for diagnosing a subject's gonadotropin abnormality.

10. The ligand of claim 9 for its use for diagnosing hypogonadotropic hypogonadism.

11. The ligand of claim 10 that binds to the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

12. The ligand of claim 10 that binds to the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

13. A method for screening a compound that affects the gonadotropic axis comprising the step of assaying the compound in the presence of a GPR54 receptor.

14. The method of claim 13, for screening for a compound that affects the LH and/or FSH secretion.

15. The method of claim 13 or 14, in which the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, or a partial protein thereof, or an ester, amide or salt thereof.

16. The method of any one of claims 13 to 15, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

17. The method of any one of claims 13 to 15, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

18. A protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

19. A protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

20. Antibodies specific to the protein of claim 18 or 19.

21. An agonist or antagonist of the GPR54 receptor for its use as an addition to a treatment for the stimulation of ovulation by GnRH.

22. An agonist or antagonist according to claim 21, wherein the agonist or antagonist positively modulates the GnRH effect on LH synthesis stimulation.

23. The agonist or antagonist of claim 21 or 22, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, or a partial protein thereof, or an ester, amide or salt thereof.

24. The agonist or antagonist of claim 21 or 22, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, from amino-acids 247 to 398.

25. The agonist or antagonist of claim 21 or 22, where the GPR54 receptor is the protein shown in SEQ ID NO:2 or SEQ ID NO:3, with the mutation L102P.

26. A composition comprising GnRH and the agonist or antagonist of claim 5 or any one of the claims 21 to 25.

27. A composition according to claim 26, wherein the agonist is the fragment 45-54 of Kiss-1.

28. A composition according to claims 26 or 27, wherein the ratio of the GnRH to the agonist or

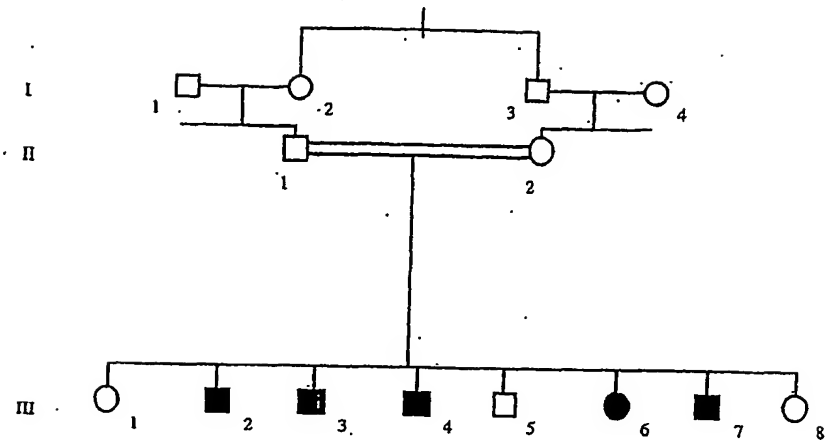
antagonist of GPR54 is in the range 10:1 to 1000:1 in Molar concentration.

ABSTRACT

The invention provides an agonist or antagonist of the GPR54 receptor for its use for treating a gonadotropin related disorder; a ligand of the GPR54 receptor for its use for diagnosing a subject's gonadotropin abnormality; a method for screening a compound that affect the gonadotropic axis comprising the step of assaying the compound in the presence of a GPR54 receptor and novel proteins useful in the above.

Figure 1

Family 1



Family 2

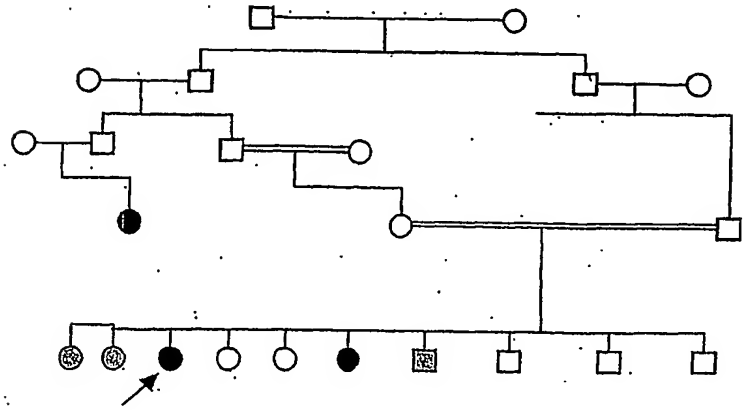


Figure 2

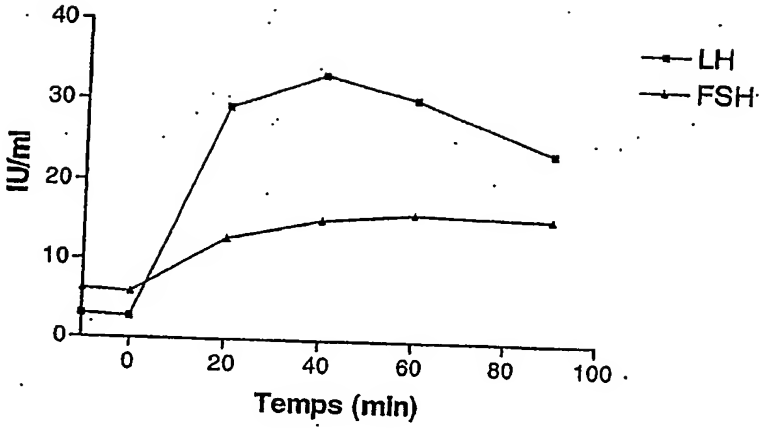


Figure 3

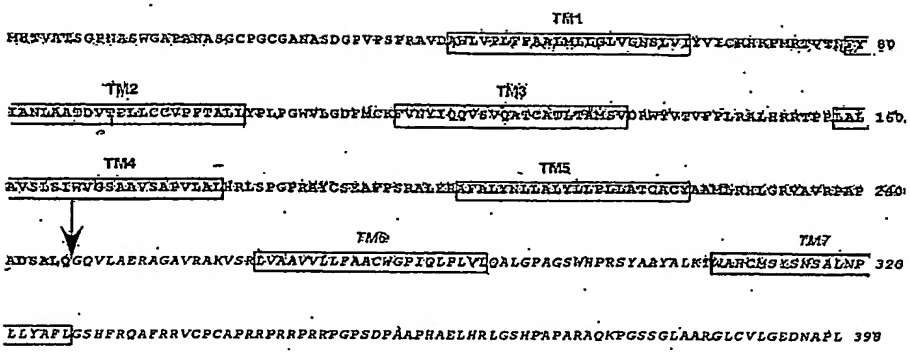
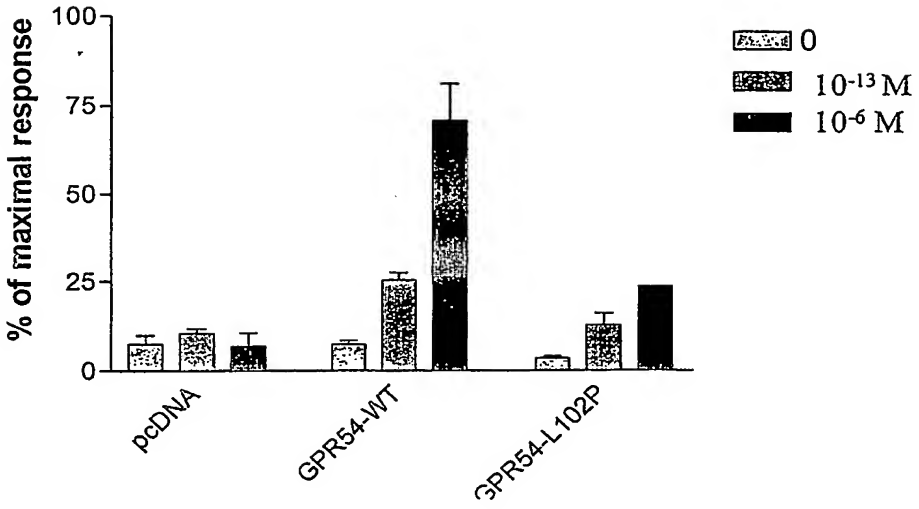


Figure 4


% of maximal response
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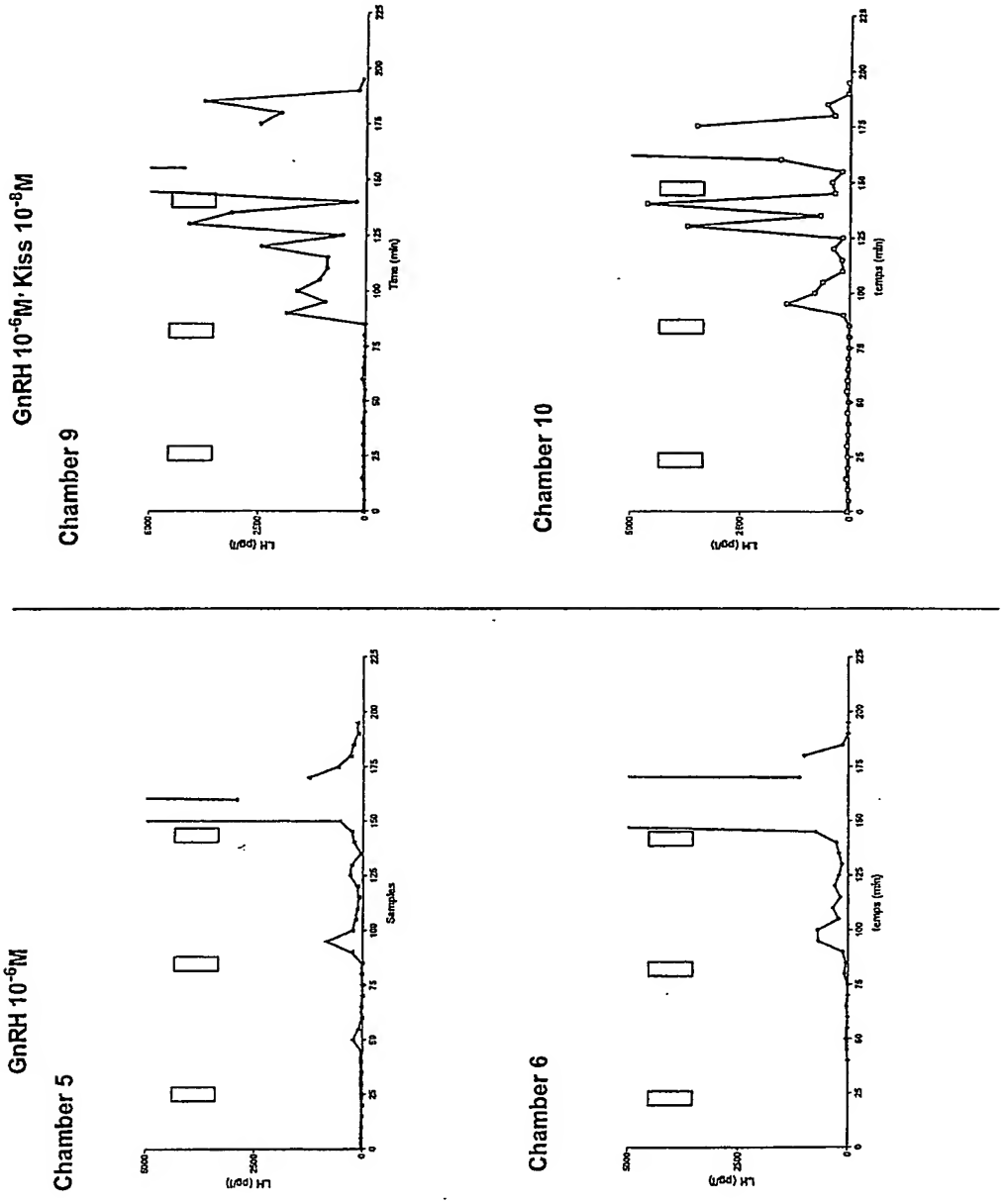
75
50
25
0
pcDNA
GPR54-WT
GPR54-L102P
0
10-13 M
10-6 M

Figure 5


GnRH 10-6M, Kiss 10-8M
Chamber 9
Chamber 10
GnRH 10-6M
Chamber 5
Chamber 6
LH (pg/l)
Time (min)
Samples
temps (min)

20990PCT GPR54.ST25
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Ala Asn Ala Ser Gly Cys Pro Gly Cys Gly Ala Asn Ala Ser Asp Gly
            20                  25                  30

cca gtc cct tcg ccg cgg gcc gtg gac gcc tgg ctc gtg ccg ctc ttc       144
Pro Val Pro Ser Pro Arg Ala Val Asp Ala Trp Leu Val Pro Leu Phe
        35                  40                  45

ttc gcg gcg ctg atg ctg ctg ggc ctg gtg ggg aac tcg ctg gtc atc       192
Phe Ala Ala Leu Met Leu Leu Gly Leu Val Gly Asn Ser Leu Val Ile
    50                  55                  60

tac gtc atc tgc cgc cac aag ccg atg cgg acc gtg acc aac ttc tac       240
Tyr Val Ile Cys Arg His Lys Pro Met Arg Thr Val Thr Asn Phe Tyr
65                  70                  75                  80

atc gcc aac ctg gcg gcc acg gac gtg acc ttc ctc ctg tgc tgc gtc       288
Ile Ala Asn Leu Ala Ala Thr Asp Val Thr Phe Leu Leu Cys Cys Val
                85                  90                  95

ccc ttc acg gcc ctg ctg tac ccg ctg ccc ggc tgg gtg ctg ggc gac       336
Pro Phe Thr Ala Leu Leu Tyr Pro Leu Pro Gly Trp Val Leu Gly Asp
            100                 105                 110

ttc atg tgc aag ttc gtc aac tac atc cag cag gtc tcg gtg cag gcc       384
Phe Met Cys Lys Phe Val Asn Tyr Ile Gln Gln Val Ser Val Gln Ala
        115                 120                 125

acg tgt gcc act ctg acc gcc atg agt gtg gac cgc tgg tac gtg acg       432
Thr Cys Ala Thr Leu Thr Ala Met Ser Val Asp Arg Trp Tyr Val Thr
    130                 135                 140

gtg ttc ccg ttg cgc gcc ctg cac cgc cgc acg ccc cgc ctg gcg ctg       480
Val Phe Pro Leu Arg Ala Leu His Arg Arg Thr Pro Arg Leu Ala Leu
145                 150                 155                 160

gct gtc agc ctc agc atc tgg gta ggc tct gcg gcg gtg tct gcg ccg       528
Ala Val Ser Leu Ser Ile Trp Val Gly Ser Ala Ala Val Ser Ala Pro
                165                 170                 175

gtg ctc gcc ctg cac cgc ctg tca ccc ggg ccg cgc gcc tac tgc agt       576
Val Leu Ala Leu His Arg Leu Ser Pro Gly Pro Arg Ala Tyr Cys Ser
            180                 185                 190

gag gcc ttc ccc agc cgc gcc ctg gag cgc gcc ttc gca ctg tac aac       624
Glu Ala Phe Pro Ser Arg Ala Leu Glu Arg Ala Phe Ala Leu Tyr Asn
        195                 200                 205

ctg ctg gcg ctg tac ctg ctg ccg ctg ctc gcc acc tgc gcc tgc tat       672
Leu Leu Ala Leu Tyr Leu Leu Pro Leu Leu Ala Thr Cys Ala Cys Tyr
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225                 230                 235                 240

gcc gat agc gcc ctg cag ggg cag gtg ctg gca gag cgc gca ggc gcc       768
Ala Asp Ser Ala Leu Gln Gly Gln Val Leu Ala Glu Arg Ala Gly Ala
                245                 250                 255
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Val Arg Ala Lys Val Ser Arg Leu Val Ala Ala Val Val Leu Leu Phe
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gcc gcc tgc tgg ggc ccc atc cag ctg ttc ctg gtg ctg cag gcg ctg      864
Ala Ala Cys Trp Gly Pro Ile Gln Leu Phe Leu Val Leu Gln Ala Leu
        275                 280                 285

ggc ccc gcg ggc tcc tgg cac cca cgc agc tac gcc gcc tac gcg ctt      912
Gly Pro Ala Gly Ser Trp His Pro Arg Ser Tyr Ala Ala Tyr Ala Leu
    290                 295                 300

aag acc tgg gct cac tgc atg tcc tac agc aac tcc gcg ctg aac ccg      960
Lys Thr Trp Ala His Cys Met Ser Tyr Ser Asn Ser Ala Leu Asn Pro
305                 310                 315                 320

ctg ctc tac gcc ttc ctg ggc tcg cac ttc cga cag gcc ttc cgc cgc     1008
Leu Leu Tyr Ala Phe Leu Gly Ser His Phe Arg Gln Ala Phe Arg Arg
                325                 330                 335

gtc tgc ccc tgc gcg ccg cgc cgc ccc cgc cgc ccc cgc cgg ccc gga     1056
Val Cys Pro Cys Ala Pro Arg Arg Pro Arg Arg Pro Arg Arg Pro Gly
            340                 345                 350

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Pro Ser Asp Pro Ala Ala Pro His Ala Glu Leu Leu Arg Leu Gly Ser
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cac ccg gcc ccc gcc agg gcg cag aag cca ggg agc agt ggg ctg gcc     1152
His Pro Ala Pro Ala Arg Ala Gln Lys Pro Gly Ser Ser Gly Leu Ala
    370                 375                 380

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Pro Val Pro Ser Pro Arg Ala Val Asp Ala Trp Leu Val Pro Leu Phe
        35                  40                  45
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Phe Ala Ala Leu Met Leu Leu Gly Leu Val Gly Asn Ser Leu Val Ile
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Tyr Val Ile Cys Arg His Lys Pro Met Arg Thr Val Thr Asn Phe Tyr
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Ile Ala Asn Leu Ala Ala Thr Asp Val Thr Phe Leu Leu Cys Cys Val
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Pro Phe Thr Ala Leu Leu Tyr Pro Leu Pro Gly Trp Val Leu Gly Asp
100 105 110

Phe Met Cys Lys Phe Val Asn Tyr Ile Gln Gln Val Ser Val Gln Ala
115 120 125

Thr Cys Ala Thr Leu Thr Ala Met Ser Val Asp Arg Trp Tyr Val Thr
130 135 140

Val Phe Pro Leu Arg Ala Leu His Arg Arg Thr Pro Arg Leu Ala Leu
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Ala Val Ser Leu Ser Ile Trp Val Gly Ser Ala Ala Val Ser Ala Pro
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Glu Ala Phe Pro Ser Arg Ala Leu Glu Arg Ala Phe Ala Leu Tyr Asn
195 200 205

Leu Leu Ala Leu Tyr Leu Leu Pro Leu Leu Ala Thr Cys Ala Cys Tyr
210 215 220

Ala Ala Met Leu Arg His Leu Gly Arg Val Ala Val Arg Pro Ala Pro
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Ala Asp Ser Ala Leu Gln Gly Gln Val Leu Ala Glu Arg Ala Gly Ala
245 250 255

Val Arg Ala Lys Val Ser Arg Leu Val Ala Ala Val Val Leu Leu Phe
260 265 270

Ala Ala Cys Trp Gly Pro Ile Gln Leu Phe Leu Val Leu Gln Ala Leu
275 280 285

Gly Pro Ala Gly Ser Trp His Pro Arg Ser Tyr Ala Ala Tyr Ala Leu
290 295 300

Lys Thr Trp Ala His Cys Met Ser Tyr Ser Asn Ser Ala Leu Asn Pro
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Leu Leu Tyr Ala Phe Leu Gly Ser His Phe Arg Gln Ala Phe Arg Arg
325 330 335

Val Cys Pro Cys Ala Pro Arg Arg Pro Arg Arg Pro Arg Arg Pro Gly
340 345 350

Pro Ser Asp Pro Ala Ala Pro His Ala Glu Leu Leu Arg Leu Gly Ser
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35 40 45

Phe Ala Ala Leu Met Leu Leu Gly Leu Val Gly Asn Ser Leu Val Ile
50 55 60

Tyr Val Ile Cys Arg His Lys Pro Met Arg Thr Val Thr Asn Phe Tyr
65 70 75 80

Ile Ala Asn Leu Ala Ala Thr Asp Val Thr Phe Leu Leu Cys Cys Val
85 90 95

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100 105 110

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210 215 220

Ala Ala Met Leu Arg His Leu Gly Arg Val Ala Val Arg Pro Ala Pro
225 230 235 240

Ala Asp Ser Ala Leu Gln Gly Gln Val Leu Ala Glu Arg Ala Gly Ala
245 250 255

Val Arg Ala Lys Val Ser Arg Leu Val Ala Ala Val Val Leu Leu Phe
260 265 270

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275 280 285

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Lys Thr Trp Ala His Cys Met Ser Tyr Ser Asn Ser Ala Leu Asn Pro
305 310 315 320

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Val Cys Pro Cys Ala Pro Arg Arg Pro Arg Arg Pro Arg Arg Pro Gly
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Pro Ser Asp Pro Ala Ala Pro His Ala Glu Leu His Arg Leu Gly Ser
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His Pro Ala Pro Ala Arg Ala Gln Lys Pro Gly Ser Ser Gly Leu Ala
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385                 390                 395
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